Exploitation of Rhizospheric Microflora for Growth of *Sorghum and Triticum*

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Exploitation of Rhizospheric Microflora for Growth Promotion of *Sorghum* and *Triticum*

ABSTRACT:

Microorganisms plays significant role in growth and yield of agricultural products to a great extent. Rhizospheric microorganisms in appropriate consortia have potential to enhance the plant growth and act as a biocontrol agent in organic farming. India is an agro based country with many issues of extensive use of agrochemicals, deteriorating soil fertility and poor yield and now gradually shifting towards organic farming. The present research proposal emphasizes on the isolation of rhizospheric microorganisms, assessment of plant growth promoting traits like nitrogen fixation, phosphate solubilization, potassium solubilization, mineralization, phytoharmones production and biocontrol roles of these isolates. Biosurfactant production of rhizospheric isolates is enhancing further plant growth promoting effect in agriculture. Among efficient isolates in all above traits will be selected. Molecular and biochemical characterization of efficient isolates, formulation of PGP microbial consortia and evaluation of their role on seed germination and plant growth by pot culture and field trials will lead to proceed to formulate bioconsortia to achieve overall growth promotion and yield improvement of *Sorghum* and *Triticum*.

INTRODUCTION:

Microorganisms are densely inhabited around plants, which can be broadly demarcated as epiphytes present on surface of plants, endophytes that are colonized inside the plant tissues, phyllospheric that grow on leaf surfaces and rhizopspheric that inhabits into the soil which is closer to roots. Rhizosphere is most dynamic and diverse among these interactions of plants and microorganisms. Generally, any soil possesses a wide and diverse microflora like bacteria, fungi, actinomycetes, algae in an approximate proportion. Beneficial microorganisms which are also referred as plant growth promoting rhizospheric bacteria (PGPR) are of critical importance in plant growth. These microorganisms help host plant to grow either directly or indirectly.

Geochemical Reactions Catalyzed by Rhizospheric Microorganisms

Rhizospheric microbiome is well involved in catabolic reactions leading to production of variety of macro and micro nutrients for plants in suitable form for them. Nitrogen, Phosphate and Potassium are always significant for plants. Various microbes are known to produce these elements for promotion of plant growth.

Nitrogen fixation and nitrification are most important mechanisms for fulfilling nitrogen requirement of plants. The examples of free-living nitrogen-fixing bacteria that are aerobic and anaerobic include *Pseudomonas*, *Azotobacter*, *Alcaligenes*, *Chlorobium*, and *Clostridium*. *Nostoc* and *Anabaena* are free-living cyanobacteria involved in nitrogen fixation.

Second most important macronutrient phosphate is solubilized by variety of microorganisms in rhizosphere. *Pseudomonas*, *Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizing bacteria in the soil (P. U. Krishnaraj *et. al.*, 2014).

Potassium is also essential macronutrient for plants which is produced by many bacteria. *Acidothiobacillus ferrooxidans*, *Paenibacillus* spp., *Bacillus mucilaginosus*, *B. edaphicus*, and *B. circulans* have potential to solubilize K minerals from immobilized form (Hassan Etesami *et. al.*, 2017).

Mineralization of various complex organic compounds into simpler form is a recognizable job of rhizospheric microbes. Soluble forms of minerals are only taken up by plants. Mineralization process is carried out by various soil microbes by degradation of complex organic compounds like cellulose, lignins, chitin, gelatin, pectin, lipids, fats, etc. Microbes possess respective enzymes catalizing degradation of above complex molecules in simpler forms (Thounaojam Thomas Meetei *et al.*, 2020).

Other mechanisms like production of siderophore for iron chelation, zink (Zn^{+2}) and sulphur solubilization and production of exopolysaccharides are also important features of beneficial rhizospheric microorganisms (Roshani *et al.*, 2020).

Production of Phytoharmones:

Several microorganisms including bacteria, fungi and actionomycetes are involved in production of plant growth promoting substances i. e. phytoharmones such as auxins, gibberlins and cytokinin. (Kanchan Vishwakarma *et. al.*, 2020). Microroganisms like *Azospirillum, Azotobactor, Flavobacterium, Psedomonas, Nocardia, Alternaria, Gibberella, Rhizopus,* etc, are known to produce phytoharmones. Elonagation of stem, induceing flowering, overcome dormancy, fruit setting, etc. are functions ofthese plant harmones. Both plants and microbes produce them and show additive effects on plantgrowth.

Influence on Nutrient Acquisition:

Most of microorganisms are known to enhance the nutrient acquisition by plants using various mechanisms. Microbes help to grasp both major and minor nutrients like nitrogen, calcium, phosphorus, magnesium etc. Arbuscular Mycorrhiza are popular in this category as they are involved in absorption of water, minerals such as phosphate, zinc, nitrogen and sulphur (Min Chen *et. at.*, 2018).

Biocontrol Activity:

Application of soil microorganisms to control plant diseases called as biological control. An eco-friendly approach of use of microbial consortia in agriculture practices is very crucial. Microorganisms produce variety of substances such as antibiotics, bacteriocins, siderophores, hydrolytic enzymes and secondary metabolites which are always important to mitigate major issue of phtophathogens. Biological control of various plant pathogens is well managed by applying various microorganisms in crop fields (Satyavir S. Sindhu *et. al.*, 2009). Antagonistic microbes do not permit growth of other microbes in proximity of plants, whereas fast growing microbes utilize all nutrients in zone so that others fail to grow there. *Trichoderma spp.*, *Pseudomonas* and *Bacillus species* are commonly used biocontrol agents in organic farming.

REVIEW OF LITERATURE:

Assessment of PGPR had been extensively studied until now on various crop plants, vegetables and cash crops. Some of them are listed as follows which shows additive effect of PGPR on plant growth promotion and protection from various phytopathogens.

The study of co-culturing beneficial microorganisms particularly phosphate and potassium solubilizing bacteria on mineral uptake and growth for pepper and cucumber showed increased mineral availability and growth for plants (H.S. Han *et al.*, 2006).

Plant growth promoting rhizobacterial (PGPR) isolates from French beans form Himachal Pradesh were studied *in-vitro* for their plant growth promoting roles and they were potent enough to use as bioferfilizers in improvement of crop yield (Ajay Kumar *et al.*, 2012).

Isolates from the Coleus rhizosphere were studied for their phytoharmone production potential and found effective in production as well as possesses growth promoting efficacy (Tisha Patel, 2017).

Potato rhizospheric isolates were assessed for nitrogen fixation and integrated nutrient uptake to enhance growth of potato *in-vitro* (Naqqash T, *et al.*, 2016).

Novel bioformulation of rhizospheric isolates along with biosurfactant produced by these isolates caused the growth promotion of *Brassica juncea* (L.) (Isha Mishra, *et al.*, 2020).

Microbial consortia over individual plant growth promoting microorganisms is crucial as they shows synergistic role of increment of all required parameters and other interactions showed increase in effectiveness in various fields (Hans C Bernstein and Ross P Carlson, 2007).

Trichoderma bio-organic fertilizer with reduced use of agrochemicals showed improvement of soil fertility and higher yield of Tomato (Lin Ye, *et al.*, 2020).

AIMS AND OBJECTIVES OF RESEARCH:

- Isolation of rhizospheric microorganisms.
- Study of rhizospheric microorganisms in terms of plant growth promoting traits.
- Study of isolates as biocontrol agents.
- Screening, extraction and structural analysis of biosurfactant and study of its role in growth promotion.
- Molecular and biochemical characterization of efficient isolates.
- Formulation of various microbial consortia by mixing number of isolates in appropriate ratios.
- Evaluation of role of these microbial consortia in seed germination and growth of plants by pot culture and field trials.
- Commercial production of bioformulations for effective application in productive agriculture.

METHODS AND MATERIALS:

1. Isolation of Microbial Cultures:

Rhizospheric soil samples are to be collected from healthy plants of Sorghum and Triticum in sterile plastic polythene bags.

Serial dilutions and streak plate technique are to be performed for isolation of soil microorganisms on solid media such as Nutrient agar, King's B agar, Potato Dextrose agar and Starch casein agar for isolation of bacteria, fungi and actinomycetes, respectfully. Isolated cultures are to be preserved into refrigerator.

2. Isolation of Phytopathogens:

Majorty of phytopathogens are soil born. They are to be isolated directly from soil or infected plant parts in fields. Nutrient agar and Potato Dextrose agar are to be used for growth respectively for bacteria and fungi. Surface disinfection with disinfectant followed by washing with distilled water to prevent contamination. Selected cultures of phytopathogens are to be stored in refrigerator for further use.

3. Evaluation of Plant Growth Promoting Traits:

3.1 Selection of nitrogen fixing isolates:

Nitrogen fixing phenomenon can be confirmed by growing the isolates on Ashby's Mannitol Agar. Only nitrogen fixers can grow on this medium due to their ability to use atmospheric nitrogen (Subba Rao, 1977).

Nitrogen-free malate medium can be used which contains bromothymol blue as an indicator for screening of nitrogen fixers. The blue colored zone around isolate indicates positive test for nitrogen fixation (R. K. Gothwal *et al.*, 2007).

Nitrogen-free glucose Mineral medium containing Bromothymol Blue (BTB) as an indicator can be used to identify nitrogen fixers by detecting blue zone around isolates (Zaw Ko Lat *et al.*, 2018).

3.2 Selection of Phosphate Solubilizers:

Phosphate solubilizating ability can be tested by using Pikovaskaya's agar medium supplemented with tricalcium phosphate; using spot inoculation method. Transparent zone around bacterial colony indicates phosphate solubilization (Pikovaskaya, 1948).

3.3 Selection of Potassium Solubilizers:

Aleksandrov medium with inclusion of an acid-base indicator dye (bromothymol blue, BTB), can be used for screening of potassium solubilizers, transparent zone around them shows potassium solubilization test positive (Rajawat, *et al.*, 2016).

3.4 Screening for Mineralization activity:

Isolates are to be tested for different activities such as cellulase, chitinase, ligninase, gelatinase and pectinase by using appropriate techniques.

3.5 Cellulase activity:

Isolates can be separately spreded on carboxymethyl cellulose medium. After 3-5 days of incubation flooding of 1% congored and 1M NaCl are to be done. Appearance of clear zone indicates cellulase activity of isolates (Shaikh N. M. *et al.*, 2013).

3.6 Chitinase activity:

Inoculation of isolates on colloidal chitin agar medium plates (1%) can be done. Positive species shows zone of clearance around them (L. Jeyanthi Rebecca, *et al.*, 2013).

3.7 Ligninase activity:

Inoculation of isolates can be done on basal medium containing 0.25 % w/v lignin. After completion of incubation period of 3-5 days plates are to be flooded with a 1 % w/v aqueous solution of $FeCl_3$ and $K_3(Fe(CN)_6)$, appearance of clear zone around colonies indicates oxidation of phenolic compounds (Stephen B. Pointing, 1999).

3.8 Gelatinase activity:

Gelatinase agar can be used to isolate gelatinase positive microorganisms. After pouring the $HgCl_2$ clear zone around the growth indicates positive test (Punam Yadav, *et al.*, 2018).

3.9 Pectinase activity:

Isolates are to be spreaded on Czapek-Dox agar medium with 1.5% pectin for screening of pectinase activity. After 3-5 days of incubation plate are to be flooded with freshly prepared Iodine-Potassium iodide solution; translucent halo zone indicates degradation of pectin in plate (Priya V, *et al.*, 2014).

4. Screening for Phytoharmones:

Production ability of important phytoharmones such as IAA, Gibberellin and Cytokinin, etc can be detected by using various standard methods as follows.

4.1 Indole acetic acid production:

24 hrs old isolate cultures are to be inoculated into tubes of Luria broth added with L-tryptophan @100µg/ml and incubation at 28^0C for 3 days. After incubation, suspension has to centrifuge at 10,000 rpm for 10 minutes. 1 ml of supernatant is to be taken into tube and addition of Salkowasky reagent for 30 minutes. Appearance of pink color indicates presence of IAA (Roshani *et al.*, 2020).

4.2 Gibberellin production:

Gibberellin production has to be assessed by method in which isolates are to be inoculated into nutrient broth and incubated for at 35^0C for 48 hrs. After incubation centrifugation of it for 15-20 minutes at 10,000 rpm are to be performed, pH has to be adjusted at 2.5 by using 3.75 N HCL. Later on supernatants are to be extracted by using liquid-liquid (ethyl acetate/$NaHCO_3$) extraction method. Gibberllin extracted into ethyl acetate phase has to measure by using UV spectrophotometer at 254 nm against control blank (N. D.Pandya, *et al.*, 2014).

4.3 Cytokinin production:

M9 medium added with 0.2% Casamino Acids, 0.01% thiamine, and 2 pg of biotin per 1000 ml has to be inoculated with 1 ml of culture for 100 ml of M9 media and incubate nearly at 28^0C on shaker at 160 rpm for 5 days. Estimation can be done on spectrophotometer at 665 nm on 72, 96 and 120 h, respectively against control blank as M9 medium (Tisha Patel, *et al.*, 2017).

5. Study of Biosurfactant Production:

Biosurfactant production can be tested by blue agar plate (BAP) test. Appearance of dark blue halo zones around spot inoculation indicates biosurfactant production.

Potential biosurfactant producers can be further grown in suitable medium for excess biosurfactant production. Extraction can be done by thin layer chromatography (TLC) method.

Further the identification of produced biosurfactants is to be completed by structural characterization of extracted product (Isha Mishra, *et al.*, 2020).

6. Study of Antipathogenic Activity *In-Vitro*:

Revival of Cultures:

Previously isolated phytopathogens from soil and infected plants are to be activated on PDA media plates for seven days. Individual rhizospheric isolates are to be activated in NB for further study.

Study is to be conducted with coculture (dual cultures) methods on PDA plates. Placing phytopathogen at center in the form of fungal agar disc and four isolates are to be added into four wells which are equidistant from center. After incubation fungal growth inhibition has to be measured against every isolate and based upon results they are to be selected for further study (B. M. Sandikar, 2008).

7. Putative Identification of Selected Isolates:

Efficient isolates for above tests are to be proceeded for putative identification by study of their biochemical characters and Morphological characters.

7.1 Study of Cultural Characteristics of Selected isolates:

Cultural study is to be studied for several characters such as color, shape, size, elevation, consistency, opacity and margin for bacterial isolates. Mycelial spreading, elevation and pigmentation are to be studied if it is fungal species.

7.2 Morphological study of selected isolates:

Morphological study can be done by using direst microscopic observation by wet mount and staining methods for fungal isolates. Morphological study for bacterial isolates is to be carried out by Gram staining method and endospore staining observation by using Schaeffer-Fulton method. Motility check is to be completed by hanging drop method.

7.3 Study of Biochemical characters of selected isolates:

General biochemical characters such as carbon source utilization, catalase activity, oxidase acitivity, amylase activity, citrate test, Vogus Proskur test and Lecithinase activity are to be studied on respective isolates (Bergey *et al.*, 1994).

8. Identification of Efficient Isolates by 16S rRNA Sequencing:

Most efficient isolates that can't be clearly identified by biochemical tests are to be genetically identified. We will proceed for commonly applied molecular techniques i. e. phylogenetic analysis of 16S r-RNA sequences by PCR method.

9. Development of Microbial Consortia:

Efficient isolates in above plant growth promoting traits and which are identified putatively and genetically can be taken as separate entity or microbial consortia in equal proportion of their active cultures. Antagonistic activity of them for each other is to be detected before formation of consortia by using co-culture method.

9.1 Assessment of Effect of Microbial consortia on Seed germination:

Developed microbial consortia are to be assessed for their effect on seed germination *in-vitro*, pot culture study and field trials. Surface disinfection of selected seeds are to be done with 0.1% mercuric choride followed by water (sterile) washing thrice. Coatings of them with microbial suspension are to be done by placing them with 24 hrs old culture for 4 hrs at 28^0 C at 100 rpm (Singh, *et al.*, 2013). Well coated seeds are to be placed in between moist towel paper and examination for their growth in incubator at 20^0C in tilted position; during incubation period of 8-10 days papers must be moistened with sterile distilled water (Roshani *et al.*, 2020).
Murashige and Skoog basal medium supplemented with 2% sucrose are to be used for the germination of microbial culture coated seeds (Mahabir Singh and Narender Singh, 2019).

Estimation of Root: Shoot ratio is to be done for determination of effect of applied microbial consortia on seed germination against control.

9.1 Pot Culture Assay:

Those microbial consortia have to be proceeded for pot culture assay which had showed good response in seed germination method.

Sterilized soils are to be used for pot culture method for different seeds. 48 hrs old microbial cultures on shaker at 80 rpm are to be centrifuged for separation of microbial cells in the form of pellets at 8000 rpm for 15 minutes in centrifuge. Re-suspensions are to be made in phosphate buffer solution (Mendoza-Hernández *et al.*, 2016). Activated cultures are to be inoculated on seeds as done in seed germination assay *in-vitro*. Controls are to be maintained without inoculation of microbial consortia in sterile soil containing pot.

Study of plant growth in presence of different microbial consortia are to be done by estimating growth performance such as shoot length, diameter of shoot, number of leaves, disease resistance and dry weight of plants in both test pots and control pots.

9.2 Field trials:

After successful study of effect of microbial consortia on plant growth both in seed germination and pot culture, field trails are to be done on actual agricultural fields for study of effectiveness, performance, stress resistance and adaptation in soil by microbial consortia. Parameters such as height of plants, diameter of stem, number of leaves, number of flowers and size of grains are to be studied.

10. Development of Bioformulations of Microbial Consortia:

After mass production of most efficient microbial consortia their bioformulations are to be developed by using inert material such as talc, peat charcoal, fecal pellets (of goat and sheep) and pressed mud. Carboxymethylcellulose (CMC) is to be used as sticking agent. Viability and shelf life are to be checked after every month for one year (B. M. Sandikar, 2008).

Liquid formulations can also be developed by using mediums like water, oil and various polymers like xanthan gum, Arabic gum, glycerol, and horticultural oils for easy, free application and lower costs (Constanza Belén Lobo *et al.*, 2019).

IMPORTANCE OF STUDY:

World's increasing population, soil, air and water pollution and health complications of farmers along with decreasing soil fertility motivates cleaner agricultural practices with sustainable strategies as solution to all above problems. Overexploitation of crops, indiscriminate use of agrochemicals leads to create instability in soil fertility. The major vision of sustainable agriculture management is to meet increasing world's food demand and preserve value of agriculture. Moreover soil microbiota plays significant role in the sustainability of agriculture in future perspective (Ugo De Corato, 2020).

We can definitely provide solution for use of agrochemicals, crop failure due to soil infertility, environmental problems. Ultimately quality improvement in farm produces which will get back more prices for farmers.

Recruitment of Microbial Consortia as alternate to chemical agents:

Sustainable agriculture is a need of hour. It is responsible for reducing dependency on chemical agents for agriculture. Intense use of single or multiple microorganisms for agriculture management is perpetual.

Significance of Microbial Inoculants over Agrochemicals:

The application of appropriate microorganisms has gained popularity over chemical and synthetic pesticides and fertilizers in agriculture management practices (Alori *et al.*, 2017).

Microbial inoculants have been intensively used in agriculture practices as an organic solution such as biopesticides or biocontrol agents, which are effective for irradiating plant pathogens and

pests. Biocontrol agents are significant in maintaining soil microbiota and ultimately fertility is also enhanced.

Likewise biofertilizers are used instead of chemical alternatives that are helpful to provide nutrients to plants such as nitrogen, phosphorus, potassium etc. Excess run off of chemical fertilizers and eutrophication, salinization of soil and generation of green house gases can be well prevented by these organic ways.

Multifold applications of microbial consortia is crucial as one can formulate consortia after pre-examination of soil by physical, chemical and biological ways, which can save abundant cost of agrochemicals and respective demerits of them by using appropriate consortia in field.

Biostimulents are also being applied in fields for the purpose of aiding nutrient assimilation by plants (Marrone, 2019). Quality and quantity of fruits, grains can be achieved by this organic solution.

Unconditional performance and longer durability of microbial inoculants leads it to the stronger contender for sustainability of agriculture.

Present Microbial Inoculants used in Agriculture:

Bioraj (Microbial Consortia Bio Growth Enhancer) is a potential bio-growth enhancer based on integration of the dynamic microbial consortia (*Bacillus and Trico derma species)* within dynamic culture media for increasing the yeild of crops grown in sodic soils as well as in normal soil. It acts as soil conditioner and biocatalyst in fields which protects crops from pathogens and also acts as growth promoter.

Arka Microbial Consortium contains N_2 Fixing, P and Zn Solubilizing and Plant Growth Promoting Microbes as a Single Formulation.

Samridhi Microbial Consortium mycorrhizal biofertilizer improves growth of the plant, builds up the population of beneficial micro-organism in the soil.

YEAR WISE WORK PLAN FOR RESEARCH WORK:

Tentative time required for research work and flow of research will be as follow.

Sr. No.	Years	Targets to be achieved
1	1st year	Intensive survey of variety of crops, climatic conditions on yield ofcrops and comparative analysis of agrochemicals verses organic solutions to improve crop yield. Rhizospheric soil collection, isolation and screening of novel isolates. Collection of large number of isolates for evaluation of plant growth promoting traits and biocontrol activity.
2	2nd year	Evaluation of various plant growth promoting traits and biocontrol activities against freshly isolated phytopathogens. Selection of potent isolates and maintenance of them for morphological and biochemical characterization. Extraction and Structural characterization of produced biosurfactant. Molecular characterization of qualified isolates.
3	3rd year	Production of microbial consortia by using selected isolates in various ratios and possible combinations after studying their interactions among themselves. Assessment of effectives of microbial consortia on seed germination, seedlings of crops and growth of crops both in pot culture and field trials.
4	4th year	Mass production of Bioformulations and study of them in actual agricultural fields. Thesis writing completion with suitable conclusions.

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